INTERNATIONAL JOURNAL OF LEPROSY

Volume 64, Number 1
Printed in the U.S.A.
(ISSN 0148-916X)

INTERNATIONAL JOURNAL OF LEPROSY
And Other Mycobacterial Diseases

VOLUME 64, NUMBER 1 MARCH 1996

Detection of *Mycobacterium leprae* in Nerve Lesions by the Polymerase Chain Reaction[1]

Philippe Chemouilli, Sally Woods,
Gerard Said, and Stewart T. Cole[2]

Leprosy, an important public health problem in many countries, is generally diagnosed by clinical examination or inspection of slit-skin smears by microscopy ([2, 5]). Bacilli are easily detected morphologically in nerve and skin biopsies of lepromatous patients. By contrast, detection of bacilli is extremely difficult in tuberculoid leprosy and in some patients under treatment or with reversal reactions. Since the causative organism *Mycobacterium leprae* has proved refractory to all attempts at *in vitro* culture, the mouse foot pad model was developed ([7]). However, mouse inoculation is a time-consuming and relatively insensitive method ([6]). Nerve lesions in the tuberculoid form of leprosy lack specificity since similar inflammatory lesions are observed in other inflammatory neuropathies, such as sarcoidosis, and chronic inflammatory and demyelinating polyneuropathy of unknown origin. For such lesions it would be extremely useful to have a sensitive method of detecting the *M. leprae* genome. In recent years, a number of laboratories have described procedures for the detection and identification of *M. leprae* that involve the polymerase chain reaction (PCR) ([3, 8, 10]). These have been applied to purified organisms or *M. leprae* in skin specimens ([1, 10]), but have not yet been tested on nerve biopsies. We describe here a simple procedure that allows the reproducible detection of *M. leprae* DNA in proteinase K-treated nerve biopsies.

MATERIALS AND METHODS

Subjects. Nerve biopsy specimens from seven lepromatous patients and three paucibacillary patients were obtained from the Institut de Léprologie Appliquée of Dakar (Senegal) and the Pavillon de Malte, Hôpital Saint-Louis, Paris. Biopsies were taken from areas of sensory loss and, in the case of paucibacillary leprosy, no skin lesions were apparent. Two of the patients had received multidrug therapy previously (further details may be found in The Table). After Ziehl-Neelsen staining, morphological studies of paraffin-embedded and semithin sections enabled us to classify sections ac-

[1] Received for publication on 17 July 1995; accepted for publication in revised form on 31 October 1995.

[2] P. Chemouilli, M.D.; G. Said, M.D., Service de Neurologie-Adulte, Centre Hospitalier Universitaire de Bicetre, 78 Rue du General Leclerc, 94275 Le Kremlin Bicetre, France. S. Woods, Ph.D.; S. T. Cole, Ph.D., Unite de Genetique Moleculaire Bacterienne, Institut Pasteur, 28 Rue du Docteur Roux, 75724 Paris 15, France.

Reprint requests to Dr. Cole at address above or Tel. 33-1-45688446; Fax. 33-1-45698593.

Present address for Dr. Woods: Pharmacia Biotech, Parc Technologique, 91898 Orsay, France.

TABLE 1. *Characteristics of leprosy patients.*[a]

Patient	Type leprosy	Type neuropathy	Previous treatment	Bacilli in nerve biopsy	Morphological findings and nerve biopsy site	Reversal reaction	No. sections positive in PCR
1 (M, 52)	TT	MM	No	No	Endoneurial sclerosis, mild inflammation Sural nerve	No	0/10
2 (M, 41)	TT	MM	No	No	Tuberculoid granuloma Superficial peroneal nerve	No	3/7
3 (M, 45)	LL	MM	Dapsone monotherapy for 1 year, 15 years before neuropathy	No	Marked endoneurial sclerosis, numerous regenerative fibers; no inflammation Superficial peroneal nerve	No	3/5
4 (M, 41)	LL	MM	3 years of MDT	No	Endo- and perineural inflammation, sclerosis and epineurial vasculitis Sural nerve	Yes (delayed)	1/3
5 (F, 42)	TT	Remittent relapsing MM	MDT, duration unknown	No	Endoneurial sclerosis with inflammatory infiltrate and epineural vasculitis Superficial peroneal nerve	No	3/7
6 (M, 38)	LL	Skin lesions + MM	No	Uncountable bacilli in cells of all nerve compartments	Inflammatory infiltrates, endoneurial sclerosis, demyelination and Superficial radial nerve	No	10/10
7 (F, 23)	LL	Skin lesions + MM	No	Uncountable bacilli in cells of all nerve compartments	Inflammatory infiltrates, endoneurial sclerosis, demyelination and Superficial radial nerve	No	10/10
8 (M, 30)	LL	Skin lesions + MM	No	Uncountable bacilli in cells of all nerve compartments	Inflammatory infiltrates, endoneurial sclerosis, demyelination and Sural nerve	No	10/10
9 (M, 27)	LL	Skin lesions + MM	Commenced MDT 2 weeks before biopsy	Uncountable bacilli in cells of all nerve compartments	Inflammatory infiltrates, endoneurial sclerosis, demyelination and Superficial radial nerve	No	10/10
10 (M, 31)	LL	Skin lesions + MM	6 months MDT 4 years previously	Uncountable bacilli in cells of all nerve compartments	Inflammatory infiltrates, endoneurial sclerosis, demyelination and Superficial radial nerve	No	10/10

[a] All patients with lepromatous leprosy (LL) had skin lesions in addition to the neuropathy which was characterized by a multifocal involvement of nerve trunks in the upper and lower extremities (MM: mononeuritis multiplex). In all of them *Mycobacterium leprae* were found in skin and nerve specimens and nerve enlargement was seen.

cording to the presence of bacilli. Two nerve specimens from patients with inflammatory lesions due to either necrotizing arteritis of the polyarteritis nodosa type or to sarcoidosis were used as negative controls.

Nerve samples. After the patient had given informed consent, nerve samples were taken by biopsy under local anesthesia and cut into three fragments: one was frozen (for PCR), one fixed in glutaraldehyde (for microscopy) and the third fixed in paraformaldehyde (PFA) (for immunology). The best PCR results were obtained when nerves were cut into ~10-μm sections using a cryostat (Frigocut, Reichert-Jung). Five to 10 sections were used for each patient. When possible, frozen and not fixed sections were used for reactions. To reduce the risk of contamination, the cryostat blades were replaced after each sample.

Preparation of samples for PCR. Sections from frozen or PFA-fixed specimens were resuspended in 100 μl of buffer (60 mM Tris HCl, pH 8.8). In the optimized protocol, sections were treated with proteinase K at 50°C for 30 min then heated for 5 min at 95°C before use for PCR. Ten-μl aliquots were used directly for PCR reactions. Proteinase K and collagenase/dispase were from Boehringer Mannheim.

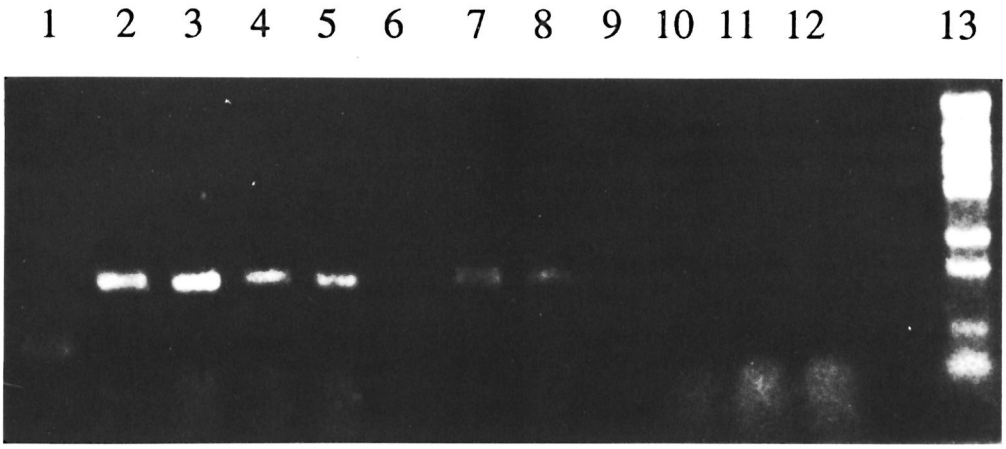
PCR reaction. Reactions were performed with primers R5 (TCACGCTTCCTGTGCTTTGC) and R6 (TGCGCTAGAAGCTTGCCGTA) directed against the *M. leprae*-specific repetitive sequence RLEP ([9]) which yielded a 447 base-pair (bp) fragment using the conditions described previously ([10]). *Taq* polymerase was purchased from Perkin Elmer Cetus. Amplification products were visualized by ethidium-bromide staining after electrophoresis of 10-μl samples on 1% agarose gels. The positive control was 0.1–10 ng of a plasmid pSW11 carrying a deleted-RLEP derivative that yields a product of 250 bp. Negative controls were reaction mixes containing all reagents but no DNA or biopsy samples from patients with necrotizing arteritis or sarcoidosis prepared by the same technique.

RESULTS

Optimization of DNA extraction from nerves. A major problem in applying PCR to the detection of *M. leprae* in nerve biopsies is the initial lysis step since the frequently extensive fibrosis of nerves, in both paucibacillary or multibacillary leprosy, makes DNA extraction very difficult. Consequently, several different lysis procedures, both mechanical and enzymatic, were attempted. In the initial experiments, PCR reactions were performed on freeze-boiled ([10]) samples, from entire nerve preparations (~3–5 mm in length), from a lepromatous leprosy patient, after mechanical teasing, with or without collagenase or collagenase/dispase treatment. The results (not shown) were poorly reproducible and collagenase/dispase was shown to contain inhibitors of *Taq* polymerase.

It was subsequently found that satisfactory results were obtained when sections of nerves were cut to a ~10-μm thickness with a cryostat and then processed for PCR. Furthermore, treatment of the nerve sections with proteinase K for 30 min prior to freeze-boiling ([10]) increased the reproducibility and sensitivity considerably. The Figure shows that when PCR was performed on serial dilutions of sections from the same nerve, with or without proteinase K treatment, the sensitivity was increased by 50- to 100-fold after proteinase K treatment.

Detection of *M. leprae* by PCR in various nerve biopsies and correlation with morphological findings. To evaluate the usefulness of this approach, the optimized extraction procedure was applied to nerve biopsies from 10 well-characterized leprosy patients with mononeuritis multiplex (The Table) who were classed in two groups. The first group (patients 6–10) represented five cases of lepromatous leprosy, and large numbers of acid-fast bacilli were visible in cells of all nerve compartments. PCR signals were generally obtained after 30 cycles of amplification with all samples tested. In the second group, which contained three tuberculoid patients and two previously treated lepromatous patients (patients 1–5), no bacilli were seen by either light or electron microscopy and 35 to 40 PCR cycles were required to generate products. However, positive results were only obtained from 4 of the 5 patients, and even then only ~35% of the sections assayed generated a PCR product. In one tuberculoid patient, all samples were negative although there was no indication of the presence of inhibitors.



THE FIGURE. Comparison of sensitivity of detection of *M. leprae* DNA in nerve specimens with or without proteinase K treatment. Frozen sural nerve sections from a lepromatous patient (#8, The Table) were suspended in Tris buffer with or without proteinase K and diluted 10- to 10^4-fold. Aliquots (10 μl) from the diluted suspension were used for PCR then analyzed by agarose gel electrophoresis. Treated and untreated samples were in lanes 2–6, and 7–11, respectively. Lane 1 = positive control (pSW11); lane 2 = undiluted; lane 3 = 10-fold dilution; lane 4 = 100-fold dilution; lane 5 = 1000-fold dilution; lane 6 = 10,000-fold dilution; lane 7 = undiluted; lane 8 = 10-fold dilution; lane 9 = 100-fold dilution; lane 10 = 1000-fold dilution; lane 11 = 10,000-fold dilution; lane 12 = negative control; lane 13 = molecular weight markers.

DISCUSSION

The type of neuropathy observed in leprosy depends upon the host's cellular immune response to *M. leprae*. Reversal reactions (type 1) may lead to further damage to the nerve without morphologically detectable bacilli. Here, a sensitive PCR-procedure for detecting *M. leprae* in nerve biopsies was developed, optimized and applied to nerve biopsies from patients with different forms of leprosy. As expected, all samples examined from lepromatous leprosy patients tested positive; whereas only 50% of the sections assayed from nerves, in which no bacilli were visible by light or electron microscopy, yielded positive results. This group included a patient presenting with reversal reactions. In the case of one tuberculoid patient, none of the samples was positive in the PCR assay.

Detection of *M. leprae* in nerves by PCR is more sensitive than by microscopy, although there is a certain variability in paucibacillary patients. This may be due to the small number of bacilli present in lesions and to the focal nature of the lesions in these patients. Our findings are consistent with the results of de Wit, *et al.* ([1]) in skin biopsies in which 56% of tuberculoid samples were PCR positive. In reversal reactions, the detection of bacterial DNA in the absence of morphologically detectable bacilli does not necessarily mean the presence of viable bacilli, since in recently treated patients, *M. leprae* DNA was found to persist long after the death of the organisms ([1, 4]). PCR may be of help to differentiate leprosy from other inflammatory neuropathies, and further studies are in progress.

SUMMARY

A simple procedure is described for the detection of *Mycobacterium leprae* by the polymerase chain reaction in nerve biopsies sectioned with a cryostat and then treated with proteinase K. All samples from lepromatous leprosy patients and the majority of samples from paucibacillary cases yielded positive results. This approach may be useful for differentiating between leprosy and other inflammatory neuropathies.

RESUMEN

Se describe un procedimiento para la detección de *Mycobacterium leprae* por la reacción en cadena de la polimerasa en secciones de biopsias de nervios pretratadas con proteinasa K. Todas las muestras de los pacientes lepromatosos y la mayoría de los pacientes pau-

cibacilares dieron resultados positivos. Este enfoque puede ser útil para diferenciar entre la lepra y otras neuropatías inflamatorias.

RÉSUMÉ

On décrit un procédé simple pour la détection de *Mycobacterium leprae* par la réaction de polymerase en chaîne dans un nerf biopsié sectionné par un cryostat et traité ensuite par proteinase K. Tous les échantillons provenant de patients lépromateux et la majorité des échantillons provenant de cas paucibacillaires ont donné des résultats positifs. Cette approche peut être utile pour différencier la lèpre d'autres neuropathies inflammatoires.

Acknowledgment. This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. Additional financial assistance was generously provided by the Association Française Raoul Follereau, the Institut National de la Santé et de la Recherche Médicale (CRE 910604 and 920813), Contrat de Recherche Clinique (910610), and the Institut Pasteur.

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